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The *WHI1*$^+$ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog

Robert Nash, George Tokiwa, Sukhjit Anand[1], Kristen Erickson and A.Bruce Futcher

PO Box 100, Cold Spring Harbor Laboratory, NY 11724, USA and [1]Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada

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***WHI1-1* is a dominant mutation that reduces cell volume by allowing cells to commit to division at abnormally small sizes, shortening the G_1 phase of the cell cycle. The gene was cloned, and dosage studies indicated that the normal gene activated commitment to division in a dose-dependent manner, and that the mutant gene had a hyperactive but qualitatively similar function. Mild over-expression of the mutant gene eliminated G_1 phase, apparently entirely relaxing the normal G_1 size control, but revealing hitherto cryptic controls. Sequence analysis showed that the hyperactivity of the mutant was caused by the loss of the C-terminal third of the wild-type protein. This portion of the protein contained PEST regions, which may be signals for protein degradation. The WHI1 protein had sequence similarity to clam cyclin A, to sea urchin cyclin and to *Schizosaccharomyces pombe* cdc13, a cyclin homolog. Since cyclins are inducers of mitosis, WHI1 may be a direct regulator of commitment to division. A probable accessory function of the WHI1 activator is to assist recovery from α factor arrest; *WHI1-1* mutant cells could not be permanently arrested by pheromone, consistent with a hyperactivation of division.**

Key words: cell cycle/cyclin/cell size/PEST hypothesis/ *WHI1*

Introduction

Coordination between growth and division is essential for all cells. This is obvious for microbes, where growth is often the sole requirement for division, but multi-cellular eukaryotes also have a basic requirement for cellular growth underlying a more complex network of signals regulating cell division. Most eukaryotic cells commit themselves to division at a particular control point, variously called the commitment point, the restriction point or Start (Pringle and Hartwell, 1981; Pardee *et al.*, 1978). In many organisms, including *Saccharomyces cerevisiae* (Johnston *et al.*, 1977), *Schizosaccharomyces pombe* (Fantes, 1977), *Chlamydomonas reinhardii* (Donnan and John, 1983), *Amoeba proteus* (Prescott, 1956) and some mammalian cells (Killander and Zetterberg, 1965; Yen *et al.*, 1985; Shields *et al.*, 1978; MacQueen and Johnson, 1983), it has been shown that growth to a critical cell volume is a necessary prerequisite for commitment. Once committed, cells inevitably complete division, and arrive back at the commitment point ready to consider another round of division. Our goal has been to discover the molecular nature of this commitment event. To this end, we have studied mutations that change the time of commitment.

In the yeasts *S.cerevisiae* and *S.pombe*, growth to critical size is the primary control on division. In *S.pombe*, for instance, division of fast-growing cells is regulated primarily at the G_2/M boundary by a size requirement for commitment to mitosis (slow-growing cells have an additional G_1 size control) (Nurse, 1975; Fantes, 1977; Nurse and Thuriaux, 1977). The *wee1*$^-$ mutation reduces the critical size required for mitosis, with the net result that *wee1*$^-$ cells are only half the volume of wild-type cells (Nurse, 1975; Nurse and Thuriaux, 1980). The *wee1*$^+$ gene has been cloned, and sequence analysis suggests that it encodes a protein kinase (Russell and Nurse, 1987). When multiple copies of *wee1*$^+$ are chromosomally integrated, cell volume increases directly in proportion to copy number (Russell and Nurse, 1987). This suggests *wee1*$^+$ is a dosage-dependent inhibitor of mitosis. It may be that the cell is titrating a constant amount of wee1$^+$ protein against an increasing volume, and only when cell growth has diluted it to a sufficiently low level can mitosis occur. If this view is correct, then wee1$^+$ may be the metric by which the proper time for mitosis is determined.

In contrast, *S.cerevisiae* has a cell cycle controlled mainly by a G_1 commitment point called Start (reviewed by Pringle and Hartwell, 1981). Attainment of a critical size is a prerequisite for Start (Johnston *et al.*, 1977; Hartwell and Unger, 1977; Jagadish and Carter, 1977; Lord and Wheals, 1980). Although many cell division cycle (*cdc*) genes have been identified that are required for Start (Hartwell *et al.*, 1973; Reed, 1980), there is no evidence that these genes regulate the time of Start; that is, they may be part of the machinery for implementing Start, as opposed to regulating it. However, a non-lethal mutation called *WHI1-1* is known to affect the time of Start; *WHI1-1* cells can initiate new cell cycles at half the volume of wild-type (i.e. *WHI1*$^+$) cells, and so are about half the volume of wild-type cells during exponential growth (Carter and Sudbery, 1980; Sudbery *et al.*, 1980). Furthermore, like *wee1*, *WHI1-1* is a co-dominant mutation, in that heterozygous diploids are intermediate in volume between homozygous mutants and homozygous wild-types (Sudbery *et al.*, 1980). This suggests that the dosage of the WHI1-1 protein is important, making it a good candidate for a molecule that might be used by the cell to titrate volume or some other signal connected with growth. Since *WHI1-1* acts in G_1 (rather than at mitosis, as *wee1*$^+$ does), we anticipated that it might play a relatively direct role in regulating cell division.

(A note on nomenclature: the mutation was originally named *whi1-1* with the expectation that it was a null mutation, and that its co-dominance was due to a dosage effect. However, we show below that the mutation is truly dominant, and so we have taken the liberty of re-naming

it *WHI1-1*, in accordance with standard *S.cerevisiae* nomenclature. We refer to the wild-type allele as *WHI1*$^+$. Deletion alleles are referred to in lower case, e.g. *whi1-310*. The protein is referred to without italics, e.g. WHI1.)

Results

Basic phenotypes of WHI1-1

The *WHI1-1* strain S673a was crossed to strain LL20, and cell size in the spore clones was assayed with a Coulter Channelyzer. After three backcrosses, the small cell phenotype always segregated 2:2 (Figure 1). The growth rates of *WHI1-1* and *WHI1*$^+$ cells were indistinguishable (data not shown). When glucose was replaced by glycerol as the carbon source, both cell types grew more slowly, and both cell types had reduced volumes (Figure 1). At low growth rates wild-type cells had a biphasic size distribution, because daughter cells were smaller than mother cells (previously budded cells). *WHI1-1* cells had a monophasic distribution because they divided relatively symmetrically at both high and low growth rates; the significance of this is not known.

Mapping, cloning and sequencing WHI1-1

Using a combination of classical mapping techniques and transposon tagging (method to be published elsewhere), we mapped *WHI1-1* to the left arm of chromosome I. *CDC24* and *WHI1-1* were very tightly linked (0 recombinants in 22 tetrads).

Kaback, Pringle and co-workers (e.g. Coleman *et al.*, 1986) mapped the transcription units near *CDC24*, calling transcription units without a known function *FUN* genes (Function Unknown Now). Our genetic data suggested that *WHI1-1* was in *FUN9*, *FUN10* or *CDC24* (Figure 2). While cloning these regions, we learned from Dr F.Cross (personal communication) that he had isolated a new mutation, *DAF1*, and mapped it to *FUN10*. *DAF1* and *WHI1-1* had very similar phenotypes, and so it seemed likely that they were allelic.

An *Apa*I–*Hpa*I fragment encompassing *FUN10* and 40 amino acids of *CYC3* was therefore cloned from a *WHI1-1* strain (Materials and methods). When this DNA (plasmid pBF30–Figure 2) was used to replace the *FUN10* locus of a wild-type strain, the strain became Whi$^-$. The same restriction fragment was also cloned from a wild-type strain and, when it was used to replace the DNA of a *WHI1-1* strain, the strain became Whi$^+$ (i.e. wild-type). An experiment described below shows definitively that *WHI1-1* is in *FUN10* rather than in *CYC3*.

Both strands of *FUN10* were sequenced (Figure 3). There is an open reading frame of 1740 nucleotides potentially

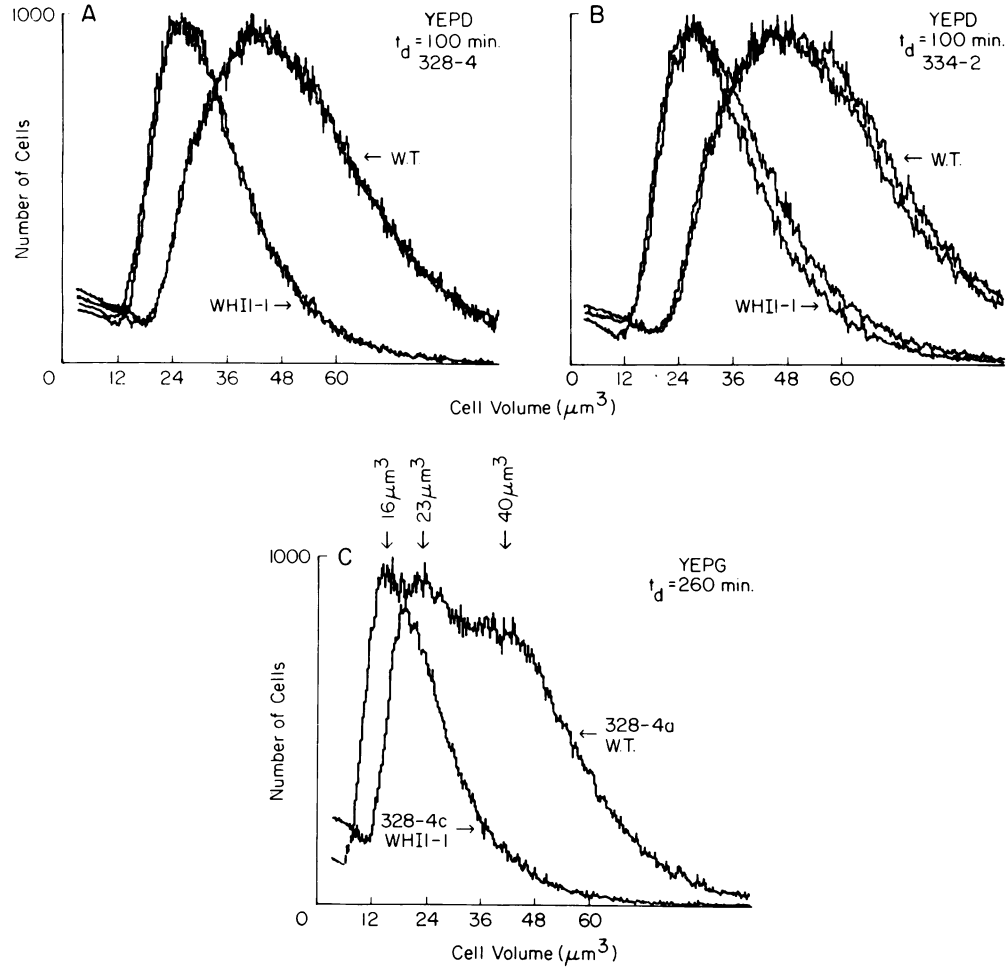

Fig. 1. Coulter Channelyzer plots of cell volume distributions. **Panel A** is tetrad BF328-4 segregating 2:2 for *WHI1-1*; **B** is tetrad BF334-2. **Panel C** shows BF328-4a (*WHI1*$^+$) and BF328-4c (*WHI1-1*) growing in YEPG (glycerol medium). t_d is the culture doubling time. The modes of the peaks are indicated in panel C.

encoding a protein of 580 amino acids. The 5′ end of the mRNA has not been mapped, but there is circumstantial evidence that the 5′-most AUG is the initiator codon. First, between the 5′-most AUG and the next in-frame AUG are 105 sense codons, a statistically improbable length for random sequence. Second, the 5′-most AUG is embedded in the sequence ACGAUGGC, which is very similar to the higher eukaryotic translation initiation consensus ACCAUGGN (Kozak, 1986), and is also similar to the consensus A(A or C)AAUGNC for yeast mRNAs (Hamilton *et al.*, 1987).

Both strands of *FUN10* from a *WHI1-1* mutant were also sequenced. The only difference found was a C to T transition at base 1210 of the open reading frame, which changes a CAG codon for glutamine to a UAG stop codon (Figure 3). This stop codon removes 177 amino acids from the carboxy terminus, but leaves the first 403 amino acids intact. Dr F. Cross (personal communication) has sequenced a different dominant *WHI1* mutation (*DAF1*), and it is also a stop codon near base 1200.

Computer searches revealed a similarity to three cyclins—sea urchin cyclin (Pines and Hunt, 1987), clam cyclin A (Swenson *et al.*, 1986) and *S.pombe* cdc13 (Booher and Beach, 1988; R.Booher, personal communication; B.Byers, personal communication). The overall percentage identity beween WHI1 and any one of the three cyclins is low; however, the percentage identity between any two of the cyclins is also rather low. The observation suggesting a family of proteins is that the three cyclins share certain small, highly conserved regions in the central parts of the proteins, and many of these regions are also found in WHI1. The region of greatest similarity is a segment of 100 amino acids stretching from residue 106 to 206 of WHI1 (KMR . . . WSI). In this segment, the three cyclins are 80–90% similar to each other (including conserved changes as well as identities), and WHI1 is just over 50% similar to the cyclin consensus (including conserved changes). When WHI1 was compared to each of the other three cyclins using only this core 100 amino acids, the IALIGN program of the Protein Identification Resource (Georgetown University, Washington, DC) gave scores that were 9–12 standard deviations from the mean, varying only slightly from cyclin to cyclin, and varying only slightly with different IALIGN parameters. Pairwise comparisons using the complete sequences were also done for us by G.Otto, using a more sophisticated statistical approach (Otto, 1986). According to this analysis, the similarities were significant at the level of 1.5×10^{-8} for WHI1 versus urchin cyclin, 4×10^{-9} for cdc13, and 6×10^{-11} for clam cyclin A. With this statistical backing, we are confident that WHI1 is a cyclin homolog, even though it is clearly less closely related to the three other cyclins than they are to each other. An alignment of the four proteins is shown in Figure 4.

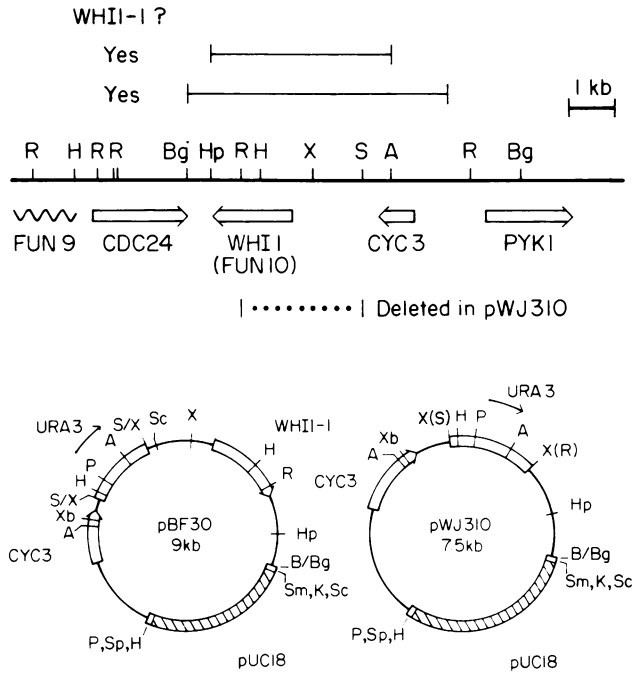

Fig. 2. Restriction map of part of chromosome I and plasmids pWJ310 and pBF30. The orientation of *FUN9* is not known. Two fragments that can transfer *WHI1-1* are shown. The deletion/disruption allele *whi1-310* has the *Eco*RI–*Sal*I chromosomal fragment replaced by the *URA3* gene, thus deleting most of the *WHI1* gene; however, the last 500 bp of the gene are retained. Other deletion/disruption alleles have the same phenotype. Restriction sites: A: *Apa*I, Bg: *Bgl*II, B/Bg: *Bam*HI/*Bgl*II hybrid, H: *Hin*dIII, Hp: *Hpa*I, K: *Kpn*I, P: *Pst*I, R: *Eco*RI, S: *Sal*I, Sc: *Sac*I, Sm: *Sma*I, Sp: *Sph*I, S/X: *Sal*I/*Xho*I hybrid, X: *Xho*I. Xb: *Xba*I, X(R): an *Eco*RI site converted to an *Xho*I site, X(S): a *Sal*I site converted to an *Xho*I site. The *Xba*I site in CYC3 is not shown in the drawing of the chromosome. Some restriction site information was adapted from Coleman *et al.* (1986), from Miyamoto *et al.* (1987) and from Gallay and Rothstein (personal communication).

Another feature of the four proteins is that they contain regions unusually rich in proline (P), glutamate (E), serine (S), threonine (T) and/or aspartate (D). It has been proposed that small regions rich in these amino acids and flanked by basic residues are specific signals for proteolysis (the PEST hypothesis—Rogers *et al.*, 1986). The PEST region in clam cyclin A has been noted previously (Swenson *et al.*, 1986). Clam and sea urchin cyclin are known to be catastrophically destroyed at mitosis, and so the idea that they contain a signal for degradation is particularly plausible. In Figure 4, the various PEST regions are underlined. Sea urchin cyclin has a relatively poor PEST region, unbounded by basic residues, but the other three proteins each have very good PEST regions. Each protein has one PEST region immediately N-terminal to the first highly conserved motif (SEY). In addition, cdc13 has multiple N-terminal PEST regions, and WHI1 has multiple C-terminal PEST regions. These latter PESTs are removed by the *WHI1-1* mutation.

A number of motifs that could allow processing of WHI1 were found; for instance, there were five occurrences of Lys Arg, two each of Arg Arg and Arg Lys and one of Lys Lys. These dibasics could allow proteolytic cleavage, and were also relatively abundant in the other three cyclins. Also, WHI1 contained eight possible glycosylation sites (Asn X Ser/Thr). Sea urchin cyclin and cdc13 also contain potential glycosylation sites. We do not yet know whether any of these potential processing sites is used.

The codon bias of *WHI1*$^+$ is 0.27, which suggests that the protein is not abundant (Bennetzen and Hall, 1982).

WHI1-1 is fully dominant, and is in the FUN10 transcription unit

A single copy of pBF30 (which carries *WHI1-1*) was integrated at the *Xho*I site of the *FUN10* locus of a wild-type

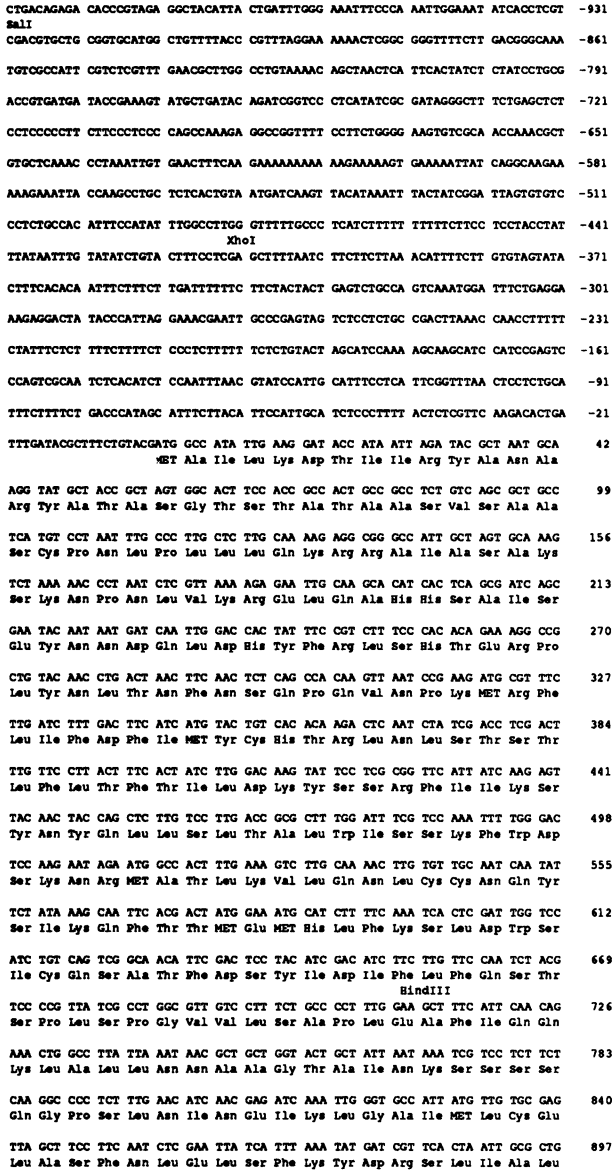

Fig. 3. The sequence of *WHI1*$^+$. The C to T transition of *WHI1-1* (base 1210) is indicated. The 5′ end of the sequence overlaps with the sequence deposited in EMBL by Dumont *et al.* (1987) for *CYC3*, and the 3′ end overlaps with the sequence of *CDC24* (Miyamoto *et al.*, 1987). Possible sites for PRTF binding (consensus TTTCCTAATTAGGAAA—Bender and Sprague, 1987) are found between nucleotides −972 and −942; −683 and −669; −292 and −277; and −118 and −103.

strain, producing transformants with one mutant and one wild-type copy of the region (*WHI1*$^+$ *WHI1-1* strains). These transformants had a volume of 27 ± 2 μm^3 (Table I), and a short G_1 (Figure 6), and were indistinguishable from *WHI1-1* mutants. This shows the mutation is fully dominant.

To show that the nonsense mutation in *FUN10* was the *WHI1-1* mutation, we removed the *Eco*RI−*Hpa*I restriction fragment carrying the C to T transition from pBF30. Cells were transformed with the remainder of pBF30. Because gap repair would replace the missing sequences with wild-type sequences (Rothstein, 1983), the transformants recovered would again have two copies of the *CYC3* and *FUN10* regions, but this time without the nonsense codon. The transformants had an average volume of 35 ± 3 μm^3, versus 42 μm^3 for their parental strain, and 27 ± 2 μm^3 for isogenic *WHI1*$^+$ *WHI1-1* transformants. Since strains with two doses of the wild-type gene (i.e. 2 × *WHI1*$^+$ strains—see below) had volumes of ~35 μm^3, this result shows that the nonsense mutation is the *WHI1-1* mutation.

WHI1 mRNA is not cell cycle regulated, but does respond to α factor

Yeast cells increase in volume as they progress through the cell cycle. When an exponentially growing culture is fractionated on the basis of cell volume, each fraction represents a different part of the cycle. Centrifugal elutriation (Hereford *et al.*, 1981) was used to separate growing cultures of *MATa/α WHI1*$^+$*/WHI1*$^+$ diploids into 11 fractions. RNA was prepared from each fraction, and subjected to Northern analysis. Each fraction contained the same proportion of *WHI1* mRNA (data not shown). Thus, the gene

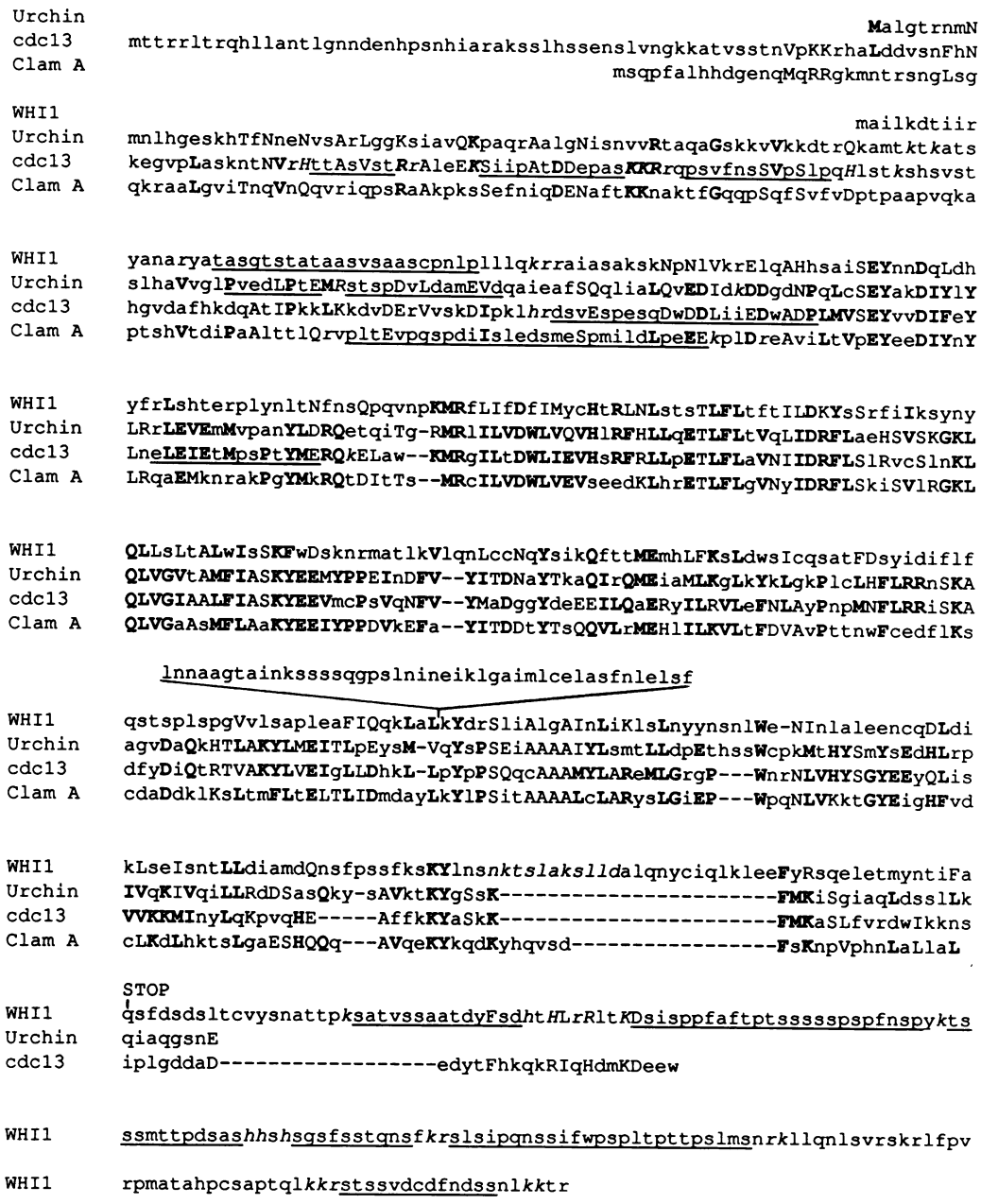

Fig. 4. Alignment of WHI1 with three cyclins. The complete sequences of WHI1, sea urchin cyclin, clam cyclin A and cdc13 are shown. Upper case letters represent conserved residues; bold upper case letters represent highly conserved residues, as defined in Materials and methods. Regions enriched in P, E, S, T and/or D (PEST regions) are underlined. Basic residues associated with the PEST regions are italicized. Also italicized is the WHI1 sequence nktslakslld, which is similar to a sequence found 95 residues more N-terminal in the other cyclins. The position of the STOP codon in *WHI1-1* is shown. Further details are given in Materials and methods.

is constitutively transcribed throughout the cycle.

To see if α factor could modulate *WHI1* expression (an experiment suggested by the α factor resistance of *WHI1-1*, see below), we treated *MATa WHI1*$^+$ and *MATa WHI1-1* cells with 5×10^{-6} M α factor for 1 h. RNA was extracted and examined by Northern analysis (Figure 5). It appeared that the *WHI1* mRNA was induced 2- to 3-fold. The amount of transcript seemed to be slightly lower in the mutant than in the wild-type. Perhaps the nonsense mutation, which would prevent translation of the last third of the message, reduces mRNA stability. In view of results presented below, it is important to note that the *WHI1-1* gene certainly does not produce significantly more mRNA than the wild-type gene.

The region upstream of *WHI1* was examined for various consensus sequences associated with α factor induction. There were several six out of eight matches to ATGAAACA or its complement (Trueheart *et al.*, 1987; Dietzel and Kurjan, 1987), but no exact copies. There were four regions with some similarity to P boxes (Bender and Sprague, 1987). A P box is thought to be the binding site for the Pheromone Recognition Transcription Factor (PRTF), which, despite its name, may be a general transcription factor (Bender and Sprague, 1987).

WHI1$^+$ *is a dose-dependent activator of Start*

In the course of other studies, B.Gallay and R.Rothstein (personal communication) constructed plasmid pWJ310

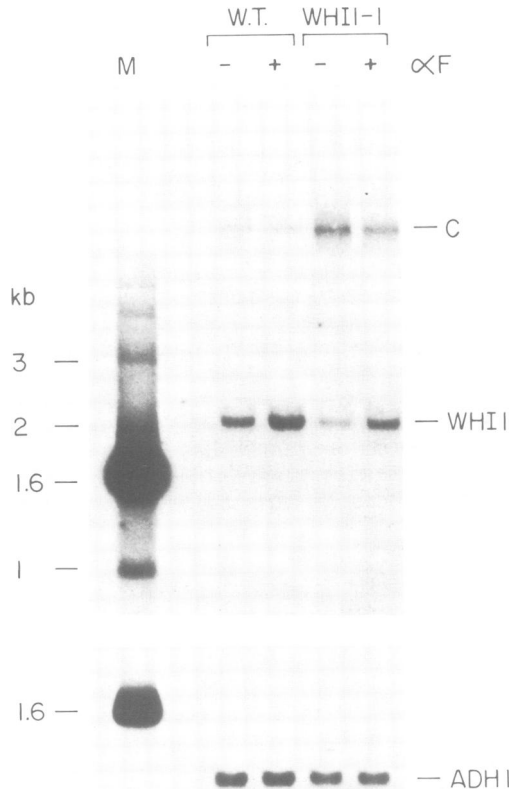

Fig. 5. The *WHI1* transcript is α factor inducible. 5 μg of total nucleic acid from a wild-type (W.T.) or *WHI1-1* strain either non-induced (−) or induced (+) for 1 h with 5×10^{-6} M α factor was electrophoresed on a 1% agarose gel, and transferred to nitrocellulose. The filter was probed with a cloned *WHI1* gene. The 2.1-kb *WHI1* transcript is indicated. The markers (lane M) are single-stranded DNA. Chromosomal DNA is indicated (C). *WHI1-1* cells, being small, have a higher DNA to RNA ratio than wild-types. After autoradiography, the probe was removed, and the filter was re-probed with cloned *ADH1* DNA as a loading control.

(Figure 2), and deleted *FUN10* in a diploid. Upon tetrad analysis, they found that *FUN10* was not an essential gene. We repeated these experiments (Materials and methods) and found that a *whi1* deletion increased cell volume by ~25%. This suggested (i) that the WHI1 protein was an activator of Start, since its absence delayed Start, making cells larger; and (ii) that the mutant WHI1-1 protein might be an over-active protein, since it had the opposite effect of a deletion.

To confirm and extend these conclusions, we surveyed the effects of over- and under-expressing the wild-type and mutant genes. This was done by integrating tandem extra copies of the genes at the chromosomal *WHI1*$^+$ locus (Materials and methods). Effects of varying *WHI1*$^+$ or *WHI1-1* expression were assayed in two ways. First, cell volumes were measured. Second, cells with DNA stained with propidium iodide were analyzed by flow cytometry to measure the lengths of G_1 and G_2 phases.

Data from many experiments are summarized in Table I, and particular experiments are shown in Figure 6. Gratifyingly, the length of G_1 was well correlated with cell volume, and *WHI1-1* did appear to act by accelerating Start, thus shortening the time cells spent in G_1. The results can be summarized as follows: (i) cells bearing a *whi1-310* deletion (the allele generated by deletion/disruption with plasmid pWJ310; Materials and methods) were ~25% larger than wild-type cells, and had the longest G_1s; (ii) 2 × *WHI1*$^+$ cells had a volume ~20% smaller than wild-type cells; i.e. a partial Whi$^-$ phenotype; (iii) *WHI1-1* cells were very small, 40% less than wild-types, and had a short G_1; and (iv) at least some 2 × *WHI1-1* cells had no visible G_1 phase, but were only marginally smaller than *WHI1-1* cells.

Table I. Effect of WHI1 alleles on cell volume and G_1 phase

Genotype	Cell volume (μm^3)	Normalized volume	% of cycle spent in G_1
whi1-310(Δ)	55 ± 5	1.2	37
WHI1$^+$	44 ± 3	1.0	27
2 × *WHI1*$^+$	35 ± 3	0.80	NT
WHI1-1	27 ± 2	0.61	19
WHI1$^+$ *WHI1-1*	27	0.61	15
2 × *WHI1-1*	25	0.57	0 to 10

NT, not tested. ± represents the range over at least eight measurements. Where ± is not indicated, less than eight measurements were done. For most constructions, each transformant behaved similarly, but for 2 × *WHI1-1* strains, some transformants repeatably had a G_1 of ~0, while others had a G_1 of ~10%.

Our results with 2 × *WHI1-1* transformants were variable. Some showed no G_1 phase, while others from the same transformation spent ~10% of their cycle in G_1. Only one 3 × and one 4 × *WHI1-1* strain were obtained; these had very short but visible G_1 phases (data not shown). The site of plasmid integration was the *Xho*I site in the *WHI1*$^+$ promoter; small alterations in the promoter may have caused variations in *WHI1-1* expression. Variations in culture conditions may also have contributed to variability.

Both alleles were also cloned into the high copy number vector YEp352 (Hill *et al.*, 1986). However, the vectors were mitotically unstable, and culture conditions had to be modified to maintain the plasmids. Because of these difficulties, the results obtained with YEp352 were not directly comparable to other results. As best we could judge, 5−10 doses of *WHI1*$^+$ resulted in a phenotype more extreme than the 2 × *WHI1*$^+$ construction, but slightly less extreme than a *WHI1-1* mutant (data not shown). Five to ten doses of *WHI1-1* shortened G_1, but did not reduce size below 27 μm^3.

All of these results are consistent with the idea that the wild-type *WHI1*$^+$ gene is a dose-dependent activator of Start, and that the mutant *WHI1-1* gene has an over-active but qualitatively similar function.

The manipulations of *WHI1*$^+$ and *WHI1-1* had surprisingly little effect on culture doubling times. Wild-type cultures typically doubled in ~90 min, while isogenic *WHI1-1* or 2 × *WHI1-1* strains doubled in 92−94 min. Thus, cells lacking any observable G_1 were nevertheless healthy. The time lost in G_1 was simply spent in some other portion of the cell cycle. We have preliminary cytological evidence that the part of G_2 immediately before nuclear division was elongated in *WHI1-1* cells.

Deleting the gene did have a small effect; *whi1-310* strains had doubling times of ~100 min, versus ~90 min for their isogenic parents, but this was a small change compared to the effect on size and G_1. Thus, G_1 can be expanded or

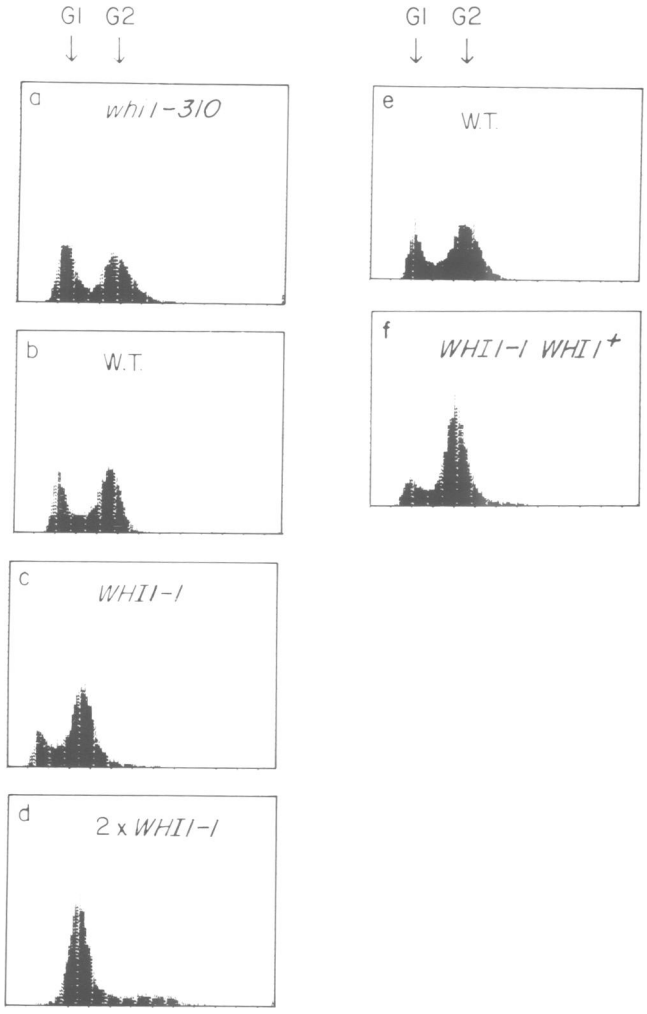

Fig. 6. Flow cytometry of mutant cells. Cells were stained with propidium iodide (Materials and methods), and the fluorescence per cell was measured. The *y* axis is the number of cells; the *x* axis is the intensity of fluorescence. Peaks due to cells in G_1 and G_2 phase are indicated. The G_2 peak actually includes cells in G_2, M and cytokinesis. The pairs of **panels a** and **b**, **c** and **d**, and **e** and **f** show parental strains and isogenic transformants. Peaks drift to the left as cells get smaller; this is an artefact due to a small amount of fluorescence from cytoplasmic cell wall material.

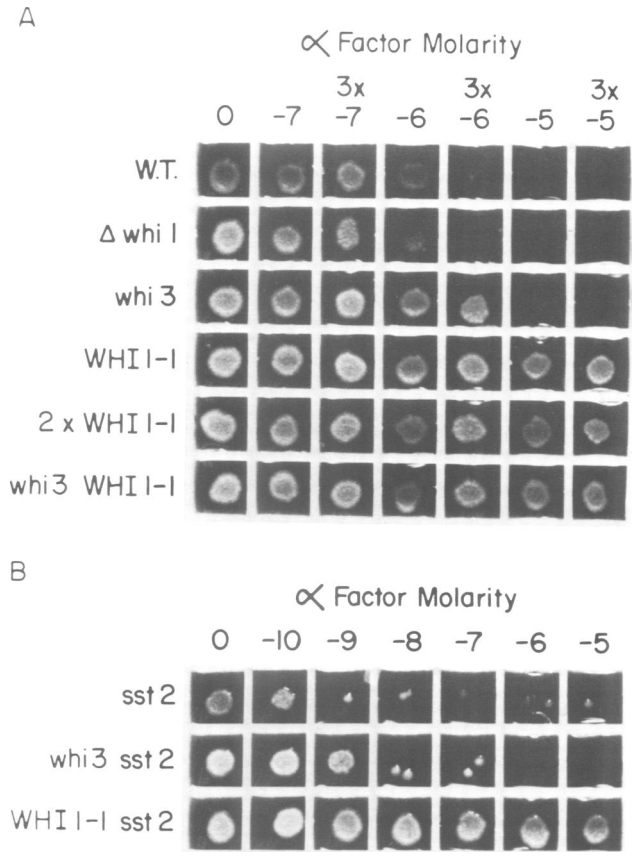

Fig. 7. α Factor resistance of mutants. **Part A**: concentrations of α factor, from left to right, are 0, 10^{-7} M, 3×10^{-7} M , 10^{-6} M, 3×10^{-6} M, 10^{-5} M, 3×10^{-5} M. Cells grew from an original inoculum of 5×10^3 cells (Materials and methods). Δwhi1 is the *whi1-310* deletion strain. Another whi mutant, *whi3*, is included for comparison. **Part B**: concentrations of α factor, from left to right, are 0, 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M. A few clonal colonies are visible in the *sst2* and *whi3 sst2* rows. These were uncharacterized resistant mutants not present in other experiments.

contracted over a wide range (0−40% of the cell cycle) without otherwise greatly affecting the cells.

WHI1-1 cells are resistant to α factor arrest

WHI1-1 × *WHI1-1* crosses often produced rather few diploids. Since *WHI1-1* seemed to activate Start, and since α factor (a yeast mating pheromone) normally inhibits Start, we wondered whether *WHI1-1* mutants might react aberrantly to α factor. We challenged *MATa WHI1-1* cells with α factor, and assayed several responses, including cell cycle arrest, mating ability and induction of the α factor-inducible mRNAs for *SST2* (Dietzel and Kurjan, 1987) and sigma (Van Arsdell *et al.*, 1987). The most striking result was that strains carrying *WHI1-1* could not be permanently arrested by α factor. While wild-type cells were permanently arrested by $\sim 3 \times 10^{-6}$ M α factor, *WHI1-1* cells efficiently formed colonies on plates containing 3×10^{-5} molar α factor (Figure 7).

Despite the lack of permanent arrest, examination by microscopy showed that when a *MATa WHI1-1* culture was first exposed to α factor the percentage of budded cells dropped, and some cells took on the characteristic shmoo shape of cycle-arrested cells. Growth curves showed a pause in division in response to pheromone, and analysis with the Channelyzer showed a large increase in cell volume shortly after application of pheromone. A G_1-less 2 × *WHI1-1* strain had a similar phenotype. For these reasons, we believe that at least some *WHI1-1* cells probably arrest when α factor is applied, but then recover quickly and continue cycling.

While *WHI1-1* cells were clearly defective in cell cycle arrest, both *MATa* and *MATα WHI1-1* cells mated reasonably well (~5-fold worse than wild-type). When treated with 5×10^{-6} M α factor, *MATa WHI1-1* cells induced the *SST2* (Figure 8) and sigma (data not shown) transcripts to the same levels as did wild-type cells. Both *MATa* and *MATα WHI1-1* cells induced shmooing in strains of the opposite mating type. Finally, *MATa/α WHI1-1/ WHI1-1* cells sporulated, albeit with a reduced frequency attributable to their small size (Calvert and Dawes, 1984). Thus, the α factor resistance phenotype seemed to be

Table II. Ability of WHI1-1 cells to enter a heat-resistant phase

Genotype	% Viability	
	Exponential cells	Stationary cells
WHI1$^+$	0.5	91
WHI1-1	2.5	96

Cells were grown to 3 × 10^7 cells/ml and then heat-treated (exponential phase), or to 2 × 10^8 cells/ml, then incubated a further 48 h, and then heat-treated (stationary phase). For heat treatment, cells were incubated at 45°C for 0 min or for 40 min, and then cells were spread on YEPD plates. The number of survivors after 40 min was compared to the 0 min control.

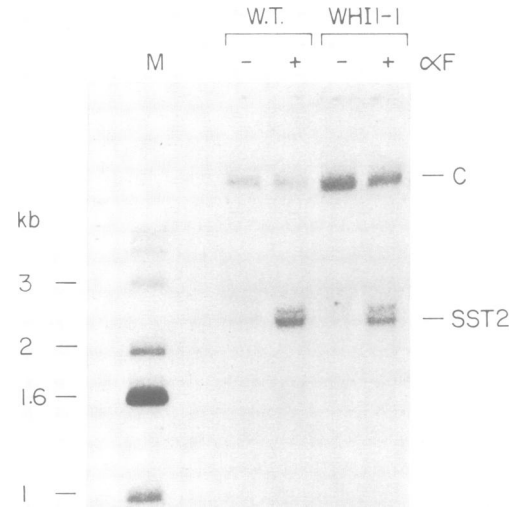

Fig. 8. *WHI1-1* strains respond to α factor induction. As Figure 4, except that the filter membrane was Nytran. The *SST2* transcript and chromosomal DNA (C) are indicated. The *SST2* transcript appears to be a doublet; this is probably a gel artefact due to the proximity of a large amount of rRNA.

specifically due to a defect in cell cycle arrest. Other α factor responses seemed normal, as did other mating functions.

The fact that α factor could make *WHI1-1* cells pause but not stop suggested that *WHI1-1* cells might recover from the effects of α factor very quickly. To address this, we examined *MATa WHI1-1 sst2* double mutants. *sst2* cells are supersensitive to α factor, and may be defective for recovery (Chan and Otte, 1982a,b). Several *MATa WHI1-1 sst2* strains were constructed (Materials and methods) and challenged with α factor. Like their parental *MATa WHI1-1 SST2* strain, they were entirely resistant to permanent arrest even by 10^{-5} M α factor (Figure 7).

Although *WHI1* influences the α factor response, it is not a haploid or mating type-specific function. *MATa, MATα* and *MATa/α* cells all show the *WHI1-1* phenotype, and the *WHI1* transcript is present in all three cell types (data not shown).

WHI1-1 cells can enter stationary phase, and require the CDC Start genes

Since *WHI1-1* cells were defective in α factor arrest, they might also be defective in other kinds of cell cycle arrest. By the criterion of heat shock resistance, *WHI1-1* cells arrested normally in stationary phase when grown to saturation in YEPD (Table II).

A number of ts lethal cell division cycle (*cdc*) mutants are known that cannot complete Start at the restrictive temperature (Reed, 1980). Double mutants between *WHI1-1* and five of the *cdc* Start genes—*cdc28-17, cdc36-16, cdc39-1, cdc61-1* and *cdc63-1*—failed to grow at 37°C, showing that Start was still part of the *WHI1-1* cell's cycle.

2 × WHI1-1 cells regain G_1 at low growth rates

When growth is slow, cells adapt by lengthening G_1 (Hartwell and Unger, 1977; Johnston *et al.*, 1977; Carter and Jagadish, 1978). It was of interest to see how G_1-less, 2 × *WHI1-1* cells would adapt to slow growth. When the mass doubling time was increased to 260 min by using glycerol as the carbon source, 2 × *WHI1-1* cultures did contain a majority of unbudded cells, i.e. cells apparently in G_1. At all growth rates, however, the proportion of unbudded cells was less in 2 × *WHI1-1* or *WHI1-1* strains than in wild-type strains (data not shown). This result suggests that even 2 × *WHI1-1* strains have a critical size for Start, but that this critical size is less than the birth size in fast-growing cultures. The critical size is greater than birth size in slow-growing cultures.

Discussion

Wild-type cells cannot bud until they have reached a critical size of ~44 μm^3. The *WHI1-1* mutation reduces this critical size to ~27 μm^3, and shortens G_1 drastically, but does not affect culture doubling time. The *WHI1-1* allele encodes a truncated version of the wild-type protein. Since complete deletion of the gene has a phenotypic effect opposite to that of the *WHI1-1* mutation, and since over-expression of the wild-type gene has an effect similar to that of *WHI1-1*, we believe that the WHI1-1 protein is a hyperactive or long-lived version of the wild-type protein with a qualitatively similar activity. The fact that cell volume and the length of G_1 are proportional to the dose of *WHI1*$^+$ (for doses between 0 and 2) is consistent with the idea that the concentration of WHI1 might be the metric by which commitment is determined.

One of our most surprising findings was that two doses of *WHI1-1* could eliminate any visible G_1 in fast-growing cells. The G_1-less cells were healthy, with apparently well coordinated cell cycles. Start has no apparent size requirement in these strains, i.e. what we had taken to be the major cell cycle control event was entirely relaxed, and yet the cells were not greatly inconvenienced, and still efficiently coordinated growth with division. This must mean that there are multiple, redundant controls preventing over-frequent cell divisions. Perhaps Start cannot occur until after cytokinesis, and this prevents overlapping cell cycles (budded buds). Also, we have preliminary cytological evidence for a control at the beginning of nuclear division. In *S.pombe*, the major cycle control point is at the beginning of nuclear division; a cryptic G_1 control is seen only under conditions of slow growth (Nurse and Thuriaux, 1977), or when the G_2/M control is relaxed by the *wee1*$^-$ mutation (Russell and Nurse, 1987). It may be that *S.pombe* and *S.cerevisiae* have controls at both points, but differ in which control is the most restrictive in fast-growing cells.

Singer and Johnston (1981) have previously argued that G_1 is dispensable in *S.cerevisiae*, and some animal cells lacking a G_1 phase have been found (Robbins and Scharff, 1967; Liskay, 1977).

Deleting *WHI1*$^+$ is not lethal. The phenotype of deletion strains argues that *WHI1*$^+$ is an activator of Start, but the fact that the cells are alive proves it is not an essential activator. Again, the most likely explanation is that there are multiple, redundant controls for activating Start. Perhaps *S.cerevisiae*, like the surf clam (Evans *et al.*, 1983), has two cyclins, and either of them is sufficient for activation of division. We are using genetic and molecular methods to find the cell cycle controls that back up *WHI1*$^+$ when it is either missing or over-active.

One other Whi mutant has been characterized. The *whi2* mutation (Sudbery *et al.*, 1980; Kelly *et al.*, 1988) makes cells small when they grow on poor carbon sources, but not when they grow on glucose medium. The mutation prevents cells from taking on stationary phase characteristics, such as heat shock resistance (Kelly *et al.*, 1988). The sequence of the gene shows no similarity to that of *WHI1* (Kelly *et al.*, 1988). *WHI1-1 whi2* double mutants are viable (Sudbery *et al.*, 1980).

Sequence analysis suggests that WHI1 is a cyclin. Cyclins were originally identified in clam and sea urchin embryos as proteins which changed in abundance as the cell cycle progressed (Evans *et al.*, 1983). The proteins are synthesized continuously, accumulate during S and early M phase, and then are catastrophically degraded near the end of M phase (Evans *et al.*, 1983). Clam cyclin A (Swenson *et al.*, 1986) and sea urchin cyclin (Pines and Hunt, 1987) have been cloned and sequenced, and mRNAs have been produced *in vitro*. When these mRNAs are injected into quiescent *Xenopus* oocytes (which are arrested in the first meiotic prophase), they push the oocytes through meiosis I. Thus, these cyclins are direct activators of meiosis, and probably mitosis (Swenson *et al.*, 1986; Pines and Hunt, 1987). *S.pombe cdc13*$^-$ mutants are blocked at the G_2/M boundary at the restrictive temperature (Booher and Beach, 1987, 1988), and this is consistent with the idea that the *cdc13*$^+$ cyclin is an activator of mitosis.

We have taken the WHI1 protein to be an activator of Start, a G_1 commitment point, while the other cyclins seem to activate an event at the G_2/M boundary. This discrepancy has several possible explanations. First, perhaps there are several classes of cyclins, and some of them act in G_1. Second, it has been argued that *S.cerevisiae* has an extended mitosis that begins at about the same time as S phase (Nurse, 1985). Perhaps the mitotic event controlled by cyclins occurs after S phase in most cell types, but at Start or shortly afterwards in *S.cerevisiae*. A third possible explanation has to do with the properties of the *cdc28* mutation (see below).

CDC28 is one of the most important proteins required for Start in *S.cerevisiae*. CDC28 has protein kinase activity when it is in a complex with several other proteins (Reed *et al.*, 1985; Mendenhall *et al.*, 1987). We have not examined the relationship between *WHI1* and *CDC28*, except to say that *WHI1-1* cannot completely suppress a *cdc28-17* mutation. In particular, we have not addressed the possibility of partial or allele-specific suppression. However, an interaction between *WHI1* and *CDC28* is to be expected for several reasons. (i) In *S.pombe*, some alleles of *cdc13*$^-$ can suppress some alleles of *cdc2*$^-$ (Booher and Beach, 1987) (*cdc2* is a *CDC28* homolog—Hindley and Phear, 1984). (ii) *cdc2*$^+$ in high copy number can suppress some alleles of *cdc13* (Booher and Beach, 1987). (iii) Cyclins are thought to activate *Xenopus* maturation promoting factor (MPF), (Swenson *et al.*, 1986; Pines and Hunt, 1987) and MPF is a complex containing the *Xenopus* homolog of the cdc2/CDC28 protein kinase (Dunphy *et al.*, 1988; Gautier *et al.*, 1988). Since the other cyclins interact with or activate their cognate CDC28 homolog, WHI1 may turn out to be an activator of CDC28 kinase activity. In this regard, it is interesting to note that Mendenhall *et al.* (1987) have postulated an 'exchangeable factor' needed for activation of the CDC28 kinase complex. This factor was apparently available only at some cell cycle stages, and so had some of the attributes of a cyclin. *CDC28* is required primarily for Start (Hartwell *et al.*, 1973; but see also Piggott *et al.*, 1982 for an alternative view), while *cdc2* is required for both Start and for mitosis (Nurse and Bissett, 1981), and MPF is only known to be required for mitosis. Therefore, if the function of a cyclin is to activate the CDC28/cdc2/MPF protein kinase, then the apparent time of action of the cyclin would be the time at which the kinase is required. Thus, in *S. cerevisiae* WHI1 would act at Start because that is where CDC28 is required, while in *Xenopus*, injected cyclins act at G_2/M because that is where MPF is required. This fails to explain why *S.pombe cdc13*$^-$ mutants do not have a Start defect.

How might WHI1 work? A speculative model is that it is synthesized continuously, and accumulates during G_1. While accumulating, it might also be modified by glycoslyation, phosphorylation, or limited proteolysis, and these modifications might increase the protein's activity. The rate of synthesis or modification might be correlated with cell size. When a sufficient amount of active WHI1 had accumulated, the cell would be pushed through Start. At some later point, the active protein would be destroyed, as other cyclins are, and the cycle of events could start again. The PEST sequences found in WHI1 and the other cyclins might be the signals for destruction; however, the idea that PEST regions are proteolytic signals has not been tested experimentally.

The effect of the *WHI1-1* mutation can be explained in the following way: the wild-type, PEST-ridden C terminus may either inhibit the wild-type protein's activity, or shorten the protein's half life. The WHI1-1 protein, which lacks the PEST tail, would therefore have a greater activity or longer half life, so that *WHI1-1* mutant cells would always have a relative excess of WHI1-1 activity. This would tend to push cells through Start prematurely, or even constitutively, as in the 2 × *WHI1-1* strains.

There are several reasons why *WHI1-1* strains might be resistant to α factor arrest. First is the possibility that the reduced cell surface area reduces the number of α factor receptors. This is unlikely for many reasons; e.g. (i) while the absolute surface area is small for a *WHI1-1* cell, the ratio of area to volume is relatively large; (ii) receptor number is not necessarily limited by area; (iii) enough signal gets through to induce other mating responses; (iv) fractional occupancy of available receptors is sufficient to arrest *sst2* cells (at 10^{-9} M α factor), but nearly full occupancy (3×10^{-5} M) does not arrest *sst2 WHI1-1* cells (the dissociation constant for α factor is $\sim 6 \times 10^{-9}$, Jenness *et al.*, 1986).

A second possibility is that cells are only sensitive to α factor arrest during a short window in G_1, and since *WHI1-1* cells have little or no G_1, they cannot be arrested (the Window of Vulnerability hypothesis). An advantage of

this model is that it correctly predicts the behavior of *whi1-301*, which has a long G_1, and is relatively sensitive to α factor (Figure 7).

A third possibility, which we favor, is that *WHI1-1* and α factor are antagonists. That is, *WHI1-1* is an activator of Start, and α factor is an inhibitor. The relative amounts of these effectors determine whether Start occurs or not. In the *WHI1-1* mutant, the activator is so powerful or abundant or long-lived that α factor is incapable of permanently inhibiting Start. We believe that this is why *WHI1-1* suppresses *sst2*.

Two observations hint that the wild-type gene may assist in normal α factor recovery. First, *WHI1*$^+$ deletion strains are ~3-fold more sensitive to α factor than wild-type strains. Second, there is some evidence that the *WHI1*$^+$ transcript is induced ~2-fold by α factor. Induction of the *WHI1*$^+$ activator would help overcome α factor-mediated inhibition of Start.

The *WHI1*$^+$ gene has many of the properties expected of a controller of cell division. Its various alleles change the volume at which commitment to division can occur, and commitment is sensitive to the concentration of the gene product. While many other genes have been identified that are clearly involved in commitment to division, to our knowledge this is the only gene identified that clearly affects the time of commitment, i.e. *WHI1*$^+$ is a regulator of commitment, not just a necessary part of the commitment machinery. With our present molecular and genetic knowledge of *WHI1*$^+$, there are several routes that can be taken to find molecules and genes that interact with it; we hope that such approaches will lead to an understanding of the molecular nature of the commitment event.

Materials and methods

Media

The media used have been described (Futcher and Carbon, 1986). YEPD buffered at pH 4.5 with 30 mM sodium succinate was used for mating assays and pheromone response tests.

Strains

Strain S673a (*MATa WHI1-1 lys2*) from P.Sudbery was crossed to strain LL20 (*MATα leu2 his3 can1*) from G.Fink. A segregant was back-crossed to LL20 three times to generate the BF328 tetrads, and four times to generate BF334 tetrads. A *his4* and a *ura3-52* marker were crossed and four times back-crossed into the BF334 background from strain BWG1-7a (*MATa ade1 ura3-52 leu2 his4*) obtained from J.Boeke. All strains used for size measurements were at least 15/16ths isogenic with strain LL20. The circular integration and transplacement techniques described by Rothstein (1983) were used to construct strains with alterations at the *WHI1* locus. Transformations were by the lithium acetate method of Ito *et al.* (1983). *WHI1*$^+$ *WHI1-1* strains were constructed by cutting the integrating plasmid pBF30 with *Xho*I, transforming a *WHI1*$^+$ *ura3* strain, and selecting for Ura$^+$. Southern analysis confirmed that most transformants had two tandem copies of the *CYC3-WHI1* region. *whi1-310* deletions were made by cutting pWJ310 with *Hpa*I and *Sph*I, transforming *WHI1*$^+$ *ura3* strains, and selecting for Ura$^+$. Southern analysis confirmed that the chromosomal region normally found between the *Sal*I and the *Eco*RI sites was missing, and had been replaced by *URA3*. 2 × *WHI1*$^+$ strains were constructed by cutting plasmid YIp352-*WHI1*$^+$ (generated by cloning a *Bgl*II chromosomal fragment carrying *WHI1*$^+$ into YIp352, Hill *et al.*, 1986) with *Xho*I, and transforming as above. 2, 3 and 4 × *WHI1-1* strains were constructed by cutting pBF30 with *Xho*I, and transforming *WHI1-1* strains. The number of integrated genes was measured by Southern analysis (see below). Size comparisons were then made between transformants and their parents, and so comparisons were between truly isogenic strains.

WHI1-1 sst2 double mutants were constructed by cutting the plasmid pBC33 (obtained from W.Courchesne and J.Thorner) with the enzyme *Sst*II, which released a disrupting fragment marked with *URA3*, and then transforming various *MATa WHI1-1* strains. To confirm that transformants were indeed *sst2* disruptants, we did Southern analysis, and also crossed the transformants to a wild-type strain. After sporulation, Whi$^+$ Sst$^-$ spore clones were recovered at the expected frequency.

Start *cdc* mutant strains were obtained from R.Singer. Strain DK17-4b (*cdc24*) was obtained from D.Kaback.

Plasmids; cloning of WHI1$^+$ and WHI1-1 DNA

Plasmid pWJ310 was obtained from B.Gallay and R.Rothstein. They constructed it by cloning an *Ava*I−*Bgl*II fragment of chromosome I into a modified (filled-in *Eco*RI site) pUC18. The *Sal*I−*Eco*RI chromosomal fragment was then removed, and replaced with a *Xho*I-linkered *URA3* fragment.

We cloned *WHI1-1* and *WHI1*$^+$ by integrating pWJ310 at the chromosomal *Hpa*I site in the *WHI1* locus. Total DNA was prepared from these transformants, digested with *Apa*I and ligated at low concentrations. The ligated DNA was used to transform *Escherichia coli* to ampicillin resistance. A plasmid consisting of pUC18 with a 5.5-kb insert of chromosome I DNA but no *URA3* gene was recovered. Of the 5.5-kb insert, some was from pWJ310, but the central 4-kb *Hpa*I−*Apa*I portion was derived from the transformed yeast strain. This was the source of DNA for most experiments, including sequence analysis.

Plasmid pBF30 was constructed by choosing one of the *WHI1-1* pUC18 clones described above, and cloning a *Xho*I-linkered *URA3* gene into the *Sal*I site in the insert.

The wild-type *WHI1*$^+$ gene was also cloned from the lambda phage C1a (Coleman *et al.*, 1986) provided by D.Kaback. A 7-kb *Bgl*II fragment carrying *WHI1*$^+$ was cloned into the integrating vector YIp352 (Hill *et al.*, 1986); this was the gene used for most but not all of the 2 × *WHI1*$^+$ constructions.

Preparation of DNA

The alkaline lysis method (Maniatis *et al.*, 1982) was used to prepare plasmids from *E.coli*. Yeast DNA was prepared as described by Holm *et al.* (1986).

Southern and Northern analysis

Southern and Northern analysis was by standard procedures (Maniatis *et al.*, 1982). The number of plasmid copies integrated at the *WHI1* locus was assayed by measuring the length of the WHI1-hybridizing *Bgl*II band after Southern analysis. *Bgl*II gives a *WHI1* chromosomal fragment of 7 kb, but does not cut within the gene or within the integrating plasmids. Because a 4 × *WHI1-1* strain produces a *Bgl*II band of ~34 kb, the gels used for analyzing potential 3 × and 4 × strains were composed of 0.5% agarose, and were run for 60 h at 1 V/cm. Resolution up to 50 kb was achieved.

Northern analysis was done on total nucleic acid. Total nucleic acid was extracted by vortexing cells with glass beads in the presence of phenol. RNA was measured by the fluorometric method of Morgan *et al.* (1979) with the modification that fluorescence was read both before and then after addition of RNase A to the assay tube so that equal quantities of RNA could be loaded in each gel lane.

Sequence analysis

WHI1-1 and *WHI1*$^+$ DNA were cloned into the single strand producing plasmids pUC118 and pUC119 (Vieira and Messing, 1987). Nested deletions were made by the method of Henikoff (1984). Single-stranded DNA was recovered with the use of the helper phage M13K07 (Vieira and Messing, 1987). Both strands of both clones were sequenced by the dideoxy method (Sanger *et al.*, 1977) with a Sequenase kit (US Biochemicals).

Computer analysis and protein alignments

Pairwise alignments between WHI1 and each of the cyclins were done using the IALIGN program of the Protein Identification Resource at Georgetown University, Washington, DC. These pairwise alignments were adjusted by eye to produce the best four-way fit. Conserved amino acid replacements were defined using the mutation data matrix by Dayhoff *et al.* (1979). The matrix gives scores ranging from −8 (W:C) to 17 (W:W) for each possible amino acid match or mis-match. The scores are based on the frequency of actual amino acid replacements between present day proteins and proteins inferred as common ancestors. In Figure 4, amino acid pairs with a score of 2 or 3 are shown in upper case, and pairs with a score of 4 or more are shown in bold upper case. Identical matches have scores between 2 and 17. Non-identities with a score of 2 or 3 are the following: R:H, R:K, R:W. N:D, N:H, D:Q, D:E, Q:E, Q:H, I:L, I:M, L:F, L:V and M:V. Non-identities with a score of 4 or more are the following: I:V, L:M and F:Y. Figure 4 was constructed by establishing a consensus sequence for the three cyclins. If two of the three had identical or conserved (conserved meaning a mutation data matrix score of two or more) amino acids at the same position, that was considered a consensus amino acid. WHI1 was then aligned with this consensus, and WHI1 amino acids were written in lower

case, upper case, or bold upper case, as appropriate to the particular match between WHI1 and the cyclin consensus.

PEST regions were found by assigning P, E, S and T a score of 2 each, D a score of 1, and all other amino acids a score of 0. The proteins were scanned with a window 15 amino acids wide. All windows with a score of 16 or more were noted and the extent of the PEST region was determined by eye, maintaining at least 50% PEST residues. Associated basic residues were found by eye. Figure 4 does not show several weak PEST regions not tightly bounded by basic residues. Note that some PEST regions are highly enriched in just one of the five PEST residues.

Measurement of cell volumes

Cells were grown in 10 ml of YEPD in a roller at 30°C to $\sim 2 \times 10^7$ cells/ml. Culture tubes were then placed in ice, and then sonicated. Cells were diluted into Isoton buffer, and cell volume was analyzed using a Coulter Counter Model ZM (70 μm aperture), and a Coulter Channelyzer Model 256 calibrated with 5.11 μm diameter plastic beads (Coulter Electronics). Volumes reported in the text are mode volumes.

Propidium iodide staining, and measurement of length of G_1 phase

Cells were grown as above, placed in ice and sonicated. 1×10^7 cells were harvested by centrifugation, and resuspended in 3 ml of water. 7 ml of 95% ethanol was added slowly while the tube was vortexed. Cells were incubated in 70% ethanol overnight at 4°C. Cells were harvested, resuspended in 5 ml of 50 mM sodium citrate, pH 7, sonicated again and then harvested and resuspended in 1 ml of the same solution. RNase A was added to a final concentration of 0.25 mg/ml, and the cells were incubated at 50°C for 1 h. 1 ml of 50 mM sodium citrate 16 μg/ml propidium iodide was added. After incubating for at least 30 min, the cells were filtered through a 36 μm mesh, and analyzed with a Coulter Model Epics-C Flow Cytometer.

To quantify the G_1 peak, we first located the modes of the G_1 and the G_2 peaks. The point midway between these modes was taken to be the midpoint of S phase. S phase was assumed to occupy 20% of the cell cycle, and so 10% of the total area under the curve was subtracted from the area to the left of the midpoint of S. Values reported in the text for G_1 were the remainder of the area left of the midpoint of S. The method's accuracy depends on whether or not S phase is truly 20% of the cycle; however, the method is objective and repeatable.

α Factor resistance assays

α Factor resistance was assayed in plastic grids, with each grid 1 cm square. Grid squares were individually filled with 1.5 ml of pH 4.5 YEPD + agar containing the appropriate concentration of α factor. After the agar had cooled and solidified, 5 μl of cell suspension containing 5×10^3 cells was spotted onto the surface. Results were scored after 2–3 days incubation. Quantitative matings were as described (Futcher and Carbon, 1986), but with a 1:1 ratio of cells.

Stationary phase analysis

The proportion of cells in stationary phase was measured as described (Plesset *et al.*, 1987), except that cells were incubated at 45°C instead of 48°C.

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References

Bender,A. and Sprague,G.F., Jr (1987) *Cell*, **50**, 681–691.
Bennetzen,J.L. and Hall,B.D. (1982) *J. Biol. Chem.*, **257**, 3026–3031.
Booher,R. and Beach,D. (1987) *EMBO J.*, **6**, 3441–3447.
Booher,R. and Beach,D. (1988) *EMBO J.*, **7**, 2321–2327.
Calvert,G.R. and Dawes,I.A. (1984) *Nature*, **312**, 61–63.
Carter,B.L.A. and Jagadish,N.N. (1978) *Exp. Cell Res.*, **112**, 373–383.
Carter,B.L.A. and Sudbery,P.E. (1980) *Genetics*, **96**, 561–566.
Chan,R.K. and Otte,C.A. (1982a) *Mol. Cell. Biol.*, **2**, 11–20.
Chan,R.K. and Otte,C.A. (1982b) *Mol. Cell. Biol.*, **2**, 21–29.
Coleman,K.G., Steensma,H.Y., Kaback,D.B. and Pringle,J.R. (1986) *Mol. Cell. Biol.*, **6**, 4516–4525.
Dayhoff,M.O., Schwartz,R.M. and Orcutt,B.C. (1979) In Dayhoff,M.O. (ed.), *Atlas of Protein Sequence and Structure*. NBRF, Washington, DC, Vol. 5, suppl. 3, pp. 345–362.
Dietzel,C. and Kurjan,J. (1987) *Mol. Cell. Biol.*, **7**, 4169–4177.
Donnan,L. and John,P.C.L. (1983) *Nature*, **304**, 630–633.
Dumont,M.E., Ernst,J.F., Hampsey,D.M. and Sherman,F. (1987) *EMBO J.*, **6**, 235–241.
Dunphy,W.G., Brizuela,L., Beach,D. and Newport,J. (1988) *Cell*, **54**, 423–431.
Evans,T., Rosenthal,E.T., Youngblom,J., Distel,J. and Hunt,T. (1983) *Cell*, **33**, 389–396.
Fantes,P.A. (1977) *J. Cell Sci.*, **24**, 51–67.
Futcher,B. and Carbon,J. (1986) *Mol. Cell. Biol.*, **6**, 2213–2222.
Gautier,J., Norbury,C., Lohka,M., Nurse,P. and Maller,J. (1988) *Cell*, **54**, 433–439.
Hamilton,R., Watanabe,C.K. and de Boer,H.A. (1987) *Nucleic Acids Res.*, **15**, 3581–3593.
Hartwell,L.H. and Unger,M.W. (1977) *J. Cell Biol.*, **75**, 422–435.
Hartwell,L.H., Mortimer,R.K., Culotti,J. and Culotti,M. (1973) *Genetics*, **74**, 267–286.
Henikoff,S. (1984) *Gene*, **28**, 351–359.
Hereford,L.M., Osley,M.A., Ludwig,J.R., II and McLaughlin,C.S. (1981) *Cell*, **24**, 367–375.
Hill,J.E., Myers,A.M., Koerner,T.J. and Tzagoloff,A. (1986) *Yeast*, **2**, 163–167.
Hindley,J. and Phear,G.A. (1984) *Gene*, **31**, 129–134.
Holm,C., Meeks-Wagner,D.W., Fangman,W.L. and Botstein,D. (1986) *Gene*, **42**, 169–173.
Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163–168.
Jagadish,M,N. and Carter,B.L.A. (1977) *Nature*, **269**, 145–147.
Jenness,D.D., Burkholer,A.C. and Hartwell,L.H. (1986) *Mol. Cell. Biol.*, **6**, 318–320.
Johnston,G.C., Pringle,J.R. and Hartwell,L.H. (1977) *Exp. Cell Res.*, **105**, 79–98.
Kelly,D.E., Trevethick,J., Mountain,H. and Sudbery,P.E. (1988) *Gene*, **66**, 205–213.
Killander,D. and Zetterberg,A. (1965) *Exp. Cell Res.*, **40**, 12–20.
Kozak,M. (1986) *Cell*, **44**, 283–292.
Liskay,R.M. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 1622–1625.
Lord,P.G. and Wheals,A.E. (1980) *J. Bacteriol.*, **142**, 808–818.
MacQueen,H.A. and Johnson,M.H. (1983) *J. Embryol. Exp. Morphol.*, **77**, 297–308.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mendenhall,M.D., Jones,C.A. and Reed,S.I. (1987) *Cell*, **50**, 927–935.
Miyamoto,S., Ohya,Y., Ohsumi,Y. and Anraku,Y. (1987) *Gene*, **54**, 125–132.
Morgan,A.R., Lee,J.S., Pulleyblank,D.E. and Evans,D.H. (1979) *Nucleic Acids Res.*, **7**, 547.
Nurse,P. (1975) *Nature*, **256**, 547–551.
Nurse,P. (1985) *Trends Genet.*, **1**, 51–55.
Nurse,P. and Bissett,Y. (1981) *Nature*, **292**, 558–560.
Nurse,P. and Thuriaux,P. (1977) *Exp. Cell. Res.*, **107**, 365–375.
Nurse,P. and Thuriaux,P. (1980) *Genetics*, **96**, 627–637.
Otto,G. (1986) Ph.D. Thesis, Massachusetts Institute of Technology.
Pardee,A.B., Dubrow,R., Hamlin,J.L. and Kletzien,R.F. (1978) *Annu. Rev. Biochem.*, **47**, 715–750.
Piggott,J.A., Rai,R. and Carter,B.L.A. (1982) *Nature*, **298**, 391–394.
Pines,J. and Hunt,T. (1987) *EMBO J.*, **6**, 2987–2995.
Plesset,J., Ludwig,J.R., Cox,B.S. and McLaughlin,C.S. (1987) *J. Bacteriol.*, **169**, 779–784.
Prescott,D.M. (1956) *Exp. Cell Res.*, **11**, 86–98.
Pringle,J.R. and Hartwell,L.H. (1981) In Strathern,J., Jones,E. and Broach,J. (eds) *The Molecular Biology of the Yeast Saccharomyces Vol. I.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Reed,S.I. (1980) *Genetics*, **95**, 561–577.
Reed,S.I., Hadwiger,J.A. and Lorincz,A.T. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4055–4059.
Robbins,E. and Scharff,M.D. (1967) *J. Cell Biol.*, **34**, 684–686.
Rogers,S., Wells,R. and Rechsteiner,M. (1986) *Science*, **234**, 364–368.
Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 202–211.
Russell,P. and Nurse,P. (1987) *Cell*, **49**, 559–567.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*,

74, 5463–5467.
Shields,R., Brooks,R.F., Riddle,P.N., Capellaro,D.F. and Delia,D. (1978) *Cell*, **15**, 469–474.
Singer,R.A. and Johnston,G.C. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 3030–3033.
Sudbery,P.E., Goodey,A.R. and Carter,B.L.A. (1980) *Nature*, **288**, 401–404.
Swenson,K.I., Farrell,K.M. and Ruderman,J.V. (1986) *Cell*, **47**, 861–870.
Trueheart,J., Boeke,J.D. and Fink,G.R. (1987) *Mol. Cell. Biol.*, **7**, 2316–2328.
Van Arsdell,S.W., Stetler,G.L. and Thorner,J. (1987) *Mol. Cell. Biol.*, **7**, 749–759.
Vieira,J. and Messing,J. (1987) *Methods Enzymol.*, **153**, 3–11.
Yen,A., Fried,J., Kitahara,T., Strife,A. and Clarkson,B.D. (1975) *Exp. Cell Res.*, **95**, 295–302.

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